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für die Biologische Sicherheit

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Position statement of the ZKBS on the risk assessment of unauthorized genetically modified petunias

Background

In the meeting of the regulatory committee under Directive 2001/18/EC on April 16, 2017 the Finnish representative reported of the discovery of unauthorized genetically modified petunias in Finland (Report of the Finnish Safety Authority EVIRA, unpublished; Bashandy and Teeri, 2017). The results of the investigations initiated by the German states´ regional supervisory authorities as well as further parties are currently being collected at the Federal Office of Consumer Protection and Food Safety, Department of Genetic Engineering. The DNA segments detected in hitherto cultured and traded petunias in Germany comprise the 35S promoter (P-35S), the nopaline synthase promoter (P-nos), the 35S terminator (T-35S), the nopaline synthase terminator (T-nos), the octopine synthase terminator (T-ocs), the kanamycin resistance gene (*nptII*), the junction element P-nos-*nptII* as well as the A1 gene from maize coding for a dihydroflavonol-4-reductase (DFR) and/or the flavonoid-3‘, 5‘-hydroxylase gene from *Petunia x hybrida*. All or some of the mentioned DNA segments were detected. The findings relate especially to orange flowering petunias, but also to other varieties. In addition, there are orange-colored petunias in which none of the DNA segments have been detected. In its letter from May 24, 2017, Germany's Federal Ministry of Food and Agriculture (BMEL) requested the Central Committee on Biological Safety (ZKBS) to conduct a risk assessment of the unauthorized genetically modified petunias identified in Germany.

According to the current state of knowledge, it can be assumed that the genetically modified petunias found on the market originate from transformation events with various plasmid constructs and therefore probably have various origins. Genetic elements and genes which are identical to the elements and genes from different plasmids were identified. The construction of these plasmids has been published. Firstly, they have been developed at the Max Planck Institute (MPI) for Plant Breeding Research in Cologne, secondly, by a working group in Japan (Meyer *et al.,* 1987, Shimada *et al.,* 1999). However, there are currently no available data which unequivocally confirm that any one of these plasmids has been used to produce the petunias found in Germany. As the precise molecular genetic characterizations

of the GM organisms are not yet available, the ZKBS can only deliver a preliminary risk assessment on this foundation.

Preliminary risk assessment:

a) Biology of the petunia

In central Europe, the petunia (*Petunia x hybrida*) has been marketed for a long time as a popular balcony plant. Its origin is South America (moderate to subtropical climate zone) where approximately 20 taxa exist that can be allocated to two groups, each displaying common features of flower morphology. Petunias belong to the nightshade family (*Solanaceae*) and are annual. The petunia which has been cultured in Europe since around 1840 presumably resulted from crossbreeding species from both taxonomic groups. A use as food or feed is unknown, however, recipes using petunia flowers as an ingredient exist. In horticulture, petunias are propagated either from seeds or cuttings. The pollen grains of petunias are mostly dispersed by moths and possibly by some other insects. Most petunias are self-compatible, however, self-incompatible varieties are also known.

b) Dispersal and establishment

Petunia seeds are not winter-hardy. Despite single reports of petunias surviving the winter (Kronenberg and Kowarik, 1989) no permanent dispersal and establishment of petunias in natural ecosystems of central Europe has been observed. In experiments carried out to increase the frost tolerance, Pennycooke *et al.* (2003) were only able to increase the frost tolerance to a lesser extent from -4 °C of the wildtype to -6 °C to -8 °C in genetically modified petunias by suppressing the enzyme α-galactosidase. A substantial increase of the frost tolerance due to unexpected effects resulting from the genetic modification to be assessed here is considered to be unlikely.

Experiments done at the MPI for Plant Breeding Research in Cologne were able to show that petunia seeds no longer germinate after an incubation in a humid medium after exposure to frost below -4 °C (MPI Köln, 1996). Petunia seeds are therefore considered as being sensitive to conditions of humid cold.

Observations from these release trials also showed that after ploughing in various petunia varieties, regardless whether they are genetically modified or not, the seeds would no longer germinate after the first frost at the latest (MPI Köln, 1996).

c) Ability to breed with wildtype relatives

Other than all other *Solanaceae* (n=12), the petunia has a set of chromosomes with n=7, making the petunia sexually incompatible with other members of this family in Europe. A transfer of pollen does not result in a cross-breeding into endemic *Solanaceae* or crops (COGEM, 2017).

d) Assessment of DNA foreign sequences

1) The *nptII* gene

The *nptII* gene is often used as a selection marker. It codes for the enzyme aminoglycoside-3'-phosphotransferase II (APH(3')II). The enzyme catalyzes the ATP-dependent phosphorylation of certain aminoglycoside antibiotics (kanamycin, neomycin, geneticin) thus rendering them inactive. A resulting resistance to antibiotics is widely spread among soil microorganisms. Kanamycin and neomycin are used in human medicine only to a limited extent. They are, however, used in veterinary medicine for a variety of indications. The enzyme possesses a high substrate specificity (Nap *et al*., 1992). Gentamicin which is of therapeutic relevance in human medicine and other aminoglycosides and aminocyclitols do not belong to its spectrum of substrates (Trieu-Cuot *et al.,* 1987; Davies, 1991; Simon und Stille, 1993). The substrate specificity of aminoglycoside-3'-phosphotransferase II suggests that no new metabolic products will be created in the genetically modified petunias under field conditions. This gene does not confer a selection advantage on the genetically modified plants under field conditions, as neither kanamycin nor neomycin prevail in the soil in concentrations detrimental to plants.

In its statement from December 2008, the ZKBS uniformly included antibiotic resistance marker genes in genetically modified plants in the safety assessment of genetically modified plants. The ZKBS came to the conclusion that events of horizontal gene transfer from genetically modified plants to other organisms, if they did occur at all, would be negligibly rare as compared to the natural processes of transmission and new formation of resistance genes and the natural presence of the resistance genes in question in natural communities of microorganisms (ZKBS, 2008). In its statement the ZKBS also stated that especially the *nptII* gene would be widely spread in soil bacteria and *Enterobacteriaceae* in Germany. Against this background, it can be assumed that the presence of the *nptII* gene in the genome of the genetically modified petunias will not take effect on the spread of this antibiotic resistance gene in the environment.

The assessment of the ZKBS comes to the same conclusion as the EFSA assessments made in the years 2004 to 2007, according to which the EFSA did not perceive any risk for

humans, animals or the environment when *nptII* is used as a marker gene in genetically modified plants.

2) Regulatory sequences

Even in the event of a transfer of the detected regulatory sequences (P-35S, P-nos, T-35S, T-nos, T-ocs) into other organisms, no relevant increase of the overall frequency of the corresponding DNA segments in the environment would occur. These regulatory sequences originate from the *Cauliflower mosaic virus* (CaMV) and *Agrobacterium tumefaciens*. CaMV is a plant-infecting, double-stranded DNA virus which is widely distributed in plants. *Agrobacterium tumefaciens* is a widely distributed soil bacterium.

3) Synthesis genes for flower pigments

Various synthesis genes for flower pigments, which were parts of recombinant constructs, were detected in the DNA samples of the examined petunias. These were either the A1 gene from *Zea mays* or the flavonoid-3‘, 5‘-hydroxylase gene from *Petunia* x *hybrida.*

3.1) The A1 gene

The first trials with genetically modified petunias were carried out at the Max Planck Institute for Plant Breeding Research in Cologne. These petunias were studied in open-field trials in 1987 and the following years. The petunias released in Cologne contained the gene A1 from *Zea mays* (Meyer *et al*., 1987). The same gene was also identified in some varieties of the discovered unauthorized petunias. The active gene product of the A1 gene is a dihydroflavonol-4-reductase (DFR), which transforms dihydrokaempferol into leukopelargonidin. The latter is further metabolized resulting in a salmon-red flower color due to the synthesis of pelargonidin. The Cologne experiments used a natural petunia mutant (RL01) for transformation which accumulates the initial substrate dihydrokaempferol because of a mutation. The petunia naturally also possesses the enzyme DFR, however, other than the maize enzyme, it cannot convert the substrate dihydrokaempferol. The introduced gene from maize was regulated by some of the control elements assessed under 2). The activity of the A1 gene in these experiments was subject to fluctuations due to internal (e.g. age) and external (e.g. abiotic environmental factors) conditions. The gene activity depended primarily on the degree of methylation of the upstream 35S promoter (Meyer *et al.*, 1992). Studies of Griesbach (1993) demonstrated that there was an increase in the production of flower pigments (anthocyanins) in genetically modified petunias as compared to non-genetically modified petunias as well as a shift of the relative proportions of the single pigment components depending on prevailing environmental factors, but also on the individual characteristics of the plant. For example, the expression rate of the introduced gene

depended on the chromosomal insertion site and on the methylation pattern. The activity rate and/or the methylation pattern do not result in any risks to the environment or to human or animal health. The enzymes and substrates of this metabolic pathway are very common in nature and exist, for example, in maize. Even in case of an unintentional consumption of realistic amounts of dihydroflavonol-4-reductase and the resulting flower pigment by animals or humans, no detrimental effects on their health are to be expected.

3.2) The flavonoid-3‘, 5‘-hydroxylase gene

This gene was amplified by means of P-35S and T-nos specific primers in DNA samples derived from the examined petunias. The nucleotide sequence of this PCR product is identical with an mRNA coding for flavonoid-3‘, 5‘-hydroxylase from *Petunia* x *hybrida*, which was isolated and expressed in petunia by a Japanese research group (Shimada *et al*., 1999). Flavonoid-3‘, 5‘-hydroxylase synthetizes 3‘, 5‘-hydroxylated anthocyanidins which are precursors of blue or pink colored flower pigments. The enzyme belongs to the cytochrome-P450 class of enzymes. In the genetically modified petunias described by Shimada *et al.* (1999) the genetic modification produced a change in the anthocyanin composition and a flower color switch from pink to magenta (light purple). Other phenotypical alterations have not been described.

The enzyme and its substrates originate from the petunia. Even in case of an unintentional consumption of realistic amounts of flavonoid-3‘, 5‘-hydroxylase and the resulting flower pigment by animals or humans, no detrimental effects on their health are to be expected.

4) Other DNA segments possibly transmitted

The DNA segments assessed in the following are parts of the transformation plasmids p35A1 and pB853, which were applied to produce genetically modified petunias at the MPI in Cologne and in Japan (Meyer *et al.*, 1987; Shimada *et al.*, 1999). Each of the two plasmids contains some of the DNA segments found in the genetically modified petunias in Germany. However, proof that p35A1 or pB853 were used for the transformation of these plants has not yet been submitted. Notwithstanding, further DNA segments of the transformation plasmids p35A1 and pB853 shall also be evaluated in the scope of this preliminary risk assessment.

The plasmid p35A1 used by the MPI in Cologne for transformation also contains the fragment from pBR322 necessary for bacterial replication and selection. It contains an origin of replication and the gene for an ampicillin resistance. On the plasmid pB853 used by Shimada *et al*., there is also a fragment derived from pBI121 which is required for bacterial replication and selection. It contains an origin of replication and the gene for a kanamycin resistance. A gene product which is functional in plants is not encoded by these sequences,

as the regulatory elements originate from bacteria. In general, the probability of a spread of these nucleic acid fragments by transmission among bacteria is by far greater than the probability of a spread by means of a horizontal gene transfer from the genetically modified plants to microorganisms.

e) Position effects, allergenicity

Owing to the insertion of the foreign genes, an impact on the expression or regulation of the plant's own genes at and/or near the insertion site might theoretically emerge and take effect on the plant's metabolic pathways. However, no observations indicating such an event have been made during the previous handling of the genetically modified petunias in Cologne in the greenhouse and in open field. In addition, mobile genetic elements (transposable elements) which could exert effects on plant genes existing at the target site by means of transposition in the genome, occur naturally in the petunias. Inactivations of genes and/or changes in the regulation of genes thus already appear in connection with point mutations, deletions or translocations. An impact of position-related integration events on the metabolic pathways of the plant is therefore possible in non-genetically modified plants as well, for which reason GM-specific effects cannot be concluded.

According to the current state of knowledge, it is impossible to make predictions on the allergic action of a protein solely on the basis of its amino acid sequence and without further analyses. However, the pollen of petunias are dispersed by wind only to a lesser degree and are not a significant cause of pollen allergies.

f) Disposal

Genetically modified petunias are currently not authorized for the European market. Therefore, when discovered, any genetically modified petunias must be destroyed. It must be ensured that the seeds of such petunias are also securely destroyed. Petunias are annual plants, they are sensitive to humid low temperatures and frost. Destruction of the plants is therefore possible if the plant material is exposed to sufficient frost inactivation (e.g. by ploughing in combination with winter dormancy). The plants can also be destroyed by composting or by the application of herbicides. However, it must taken into account that herbicides often fail to kill off the plants' seeds. Measures such as composting will succeed in destroying the seeds if the plant material is exposed to a temperature of 60°C for several days. In addition, methods of combustion and autoclaving are also suitable. However, autoclaving is not universally applicable because of the technical quantitative limitation.

Summary

The petunia is an annual and cold-sensitive plant. There are no indications for the establishment of petunias in the environment or a cross-breeding into wildtype relatives. If genetically modified petunias were produced by a transformation with the plasmids p35A1 or pB853 or plasmids with identical genes and regulatory elements, these plants will not differ from conventional plants in regards to their risks to humans, animals and the environment. Even if the above-mentioned DNA segments were not derived from transformation events with the plasmids p35A1 or pB853, based on the available data there are no indications that genetically modified petunias harbor a higher risk than conventional petunias do.

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